Design and implementation of a rapid-mixer flow cell for time-resolved infrared microspectroscopy

Nebojsa S. Marinkovic,[a] Aleksandar R. Adzic, and Michael Sullivan
Center for Synchrotron Biosciences and Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 11461

Kevin Kovacs
Center for Synchrotron Biosciences, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 11461

Lisa M. Miller
National Synchrotron Light Source, Brookhaven National Laboratory, Upton, New York 11973

Denis L. Rousseau and Syun-Ru Yeh
Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 11461

Mark R. Chance[a]
Center for Synchrotron Biosciences and Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 11461

(Received 2 May 2000; accepted for publication 26 June 2000)

A rapid mixer for the analysis of reactions in the millisecond and submillisecond time domains by Fourier-transform infrared microspectroscopy has been constructed. The cell was tested by examination of cytochrome-*c* folding kinetics. The device allows collection of full infrared spectral data on millisecond and faster time scales subsequent to chemical jump reaction initiation. The data quality is sufficiently good such that spectral fitting techniques could be applied to analysis of the data. Thus, this method provides an advantage over kinetic measurements at single wavelengths using infrared laser or diode sources, particularly where band overlap exists. © *2000 American Institute of Physics.* [S0034-6748(00)04911-X]

I. INTRODUCTION

The progression of rapid reactions has been followed using a number of different techniques that are sensitive to protein structure, such as circular dichroism, fluorescence, pulsed H-exchange labeling, infrared, and Raman spectroscopies.[1–8] Many protein-folding studies utilize these rapid-reaction techniques to observe the time-resolved kinetics of the folding process following an external trigger into a metastable phase. Kinetics of reactions on time scales as fast as tens of microseconds have been observed subsequent to mixing two solutions in a rapid-mixer flow cell utilizing fluorescence or Raman spectroscopy as a probe.[6,7,9] In this article we describe the modification of this basic flow cell design, described previously,[6] to permit its use in conjunction with a synchrotron based infrared microscope. This technique allows the collection of full Fourier-transform infrared (FTIR) data from samples during reactions at time scales down to milliseconds or even microseconds.

II. CELL DESIGN

The cell consists of a rapid mixer in which the two reacting solutions are mixed and a long, narrow channel that forms the observation window (Fig. 1). The mixer was designed previously.[6] It consists of a 100 μm wide, 100 μm deep channel engraved in a stainless steel block. The dead time of this mixer has been previously shown to be 100 μs.[6] The channel in the observation cell was fabricated by cutting a 50 μm deep, 150–200 μm wide groove in one of two ZnSe windows using a custom-made apparatus (see later). The depth of 50 μm is selected to permit sufficient transmission of the infrared light through the solvent. The grooved window is sandwiched with a flat window. The solutions are pushed into the mixing well using a dual syringe pump (Harvard Apparatus, model 33). The resulting mixed solution flows through a custom-made dual Durometer seal (0.4 mm thick), the channel, and exits through an O ring that seals the windows to the cell into an outlet collection vessel. An infrared beam imaged through a commercial FTIR spectrometer (Nicolet Magna 860) from a synchrotron source is focused onto a point inside the observation channel using an infrared microscope (Nicolet NicPlan), and the spectral collection is initiated using steady state scanning parameters of the FTIR spectrometer. By collecting data at various positions along the channel, one obtains the spectrum at various time points after mixing and can examine the time-resolved reaction kinetics as well as the full spectrum at each point. The synchrotron radiation source is chosen because its brightness is 100–1000 times that of thermal sources,[10,11] so that sufficient energy irradiates the sample when the beam is focused onto a very narrow spot close to the diffraction limit

[a]Authors to whom correspondence should be addressed; electronic mail: marinkov@bnl.gov, mrc@aecom.yu.edu

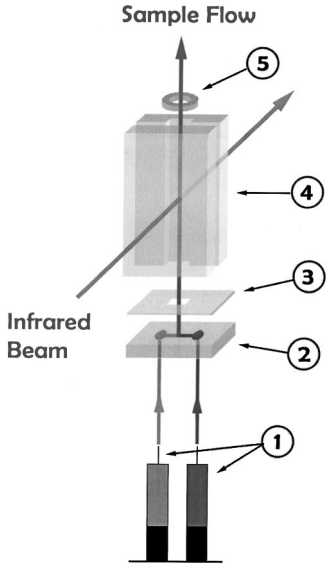

FIG. 1. Schematic of the rapid-mixer flow cell: (1) syringes with solutions, (2) mixing block with a 100 μm deep, 100 μm wide mixing well, (3) 0.4-mm-thick dual Durometer seal, (4) observation window with 50 μm deep, 200 μm wide channel in one of the two ZnSe windows, and (5) O ring that seals the cell to the output tube.

(5 μm at 2000 cm^{-1}). A single wavelength infrared laser or diode source could also provide a probe for this experiment. However, the advantage of the broadband synchrotron beam (as seen later) is the ability to collect full spectra at high signal-to-noise ratio so that spectral fitting of a band into its components is possible.

III. GROOVE CUTTING AND POLISHING

To ensure a precise depth of the optical path inside the cell, a cut groove was chosen over a window-spacer-window sandwich arrangement because the latter can suffer higher swelling at high solution pressures. ZnSe windows were chosen because this material combines (1) a good mechanical stability so that, unlike other water-insoluble windows (CaF_2, BaF_2, and KRS-5), it resists chipping while the groove is being cut, (2) a resistance to acidic media, and (3) transparency over a relatively wide mid-IR range. Also, in order to be able to easily align the microscope objective on the observation channel, transparency in the visible range is desirable.

Groove cutting was performed as follows: both sides of ZnSe windows were polished with alumina powder down to a flatness of 0.05 μm to ensure a good seal between the two crystals after the cell is assembled and to achieve a channel with a uniform depth. The flatness of the crystals was tested with an interferometer (Optical Flat, Edmund Scientific) to be better than 0.1 μm. One crystal was attached to a custom-made, computer-controlled x–z motion table. A custom-made 120-μm-thick brass disk mounted between two 1-mm-thick stainless steel supports and rotating at 5000 rpm, served as the cutting tool. The window is lifted by the x–z table towards the cutter, 5 μm at a time, while cutting is undertaken by a full length (25 mm) travel along the x axis at about 0.2 $mm\,s^{-1}$, until the required depth was obtained (e.g., 10 cuts were made). Before each sweep, a small amount of a 1 μm grade diamond paste (Buehler) was applied on the window to ensure that cutting occurred without chipping. The groove depth was uniform within 2 μm, as checked by an optical microscope. The cross section of the cut is oval, and its cross-sectional area deviates from a square shape by about 15%. The deviation does not affect the measurement because the IR beam size is much smaller than the width of the groove, so that an area of a constant depth can be easily found.

IV. PERFORMANCE

Since the dimension of the groove and the flow rate of the solution are known, one can easily calculate the time that it takes the solution to reach a specified point in the cell downstream from the origin of mixing. For instance, for a 50×200 μm groove, the flow rate of 1.0 $ml\,min^{-1}$ corresponds to linear solution speed of 2 $m\,s^{-1}$ (taking into account the reduction in the area cross section), so that each millimeter along the groove corresponds to an increment of 0.5 ms in time past the origin point. Swelling of the cell was tested by comparing the Amide I band intensities at various flow rates with that of a stationary solution in the cell, and found to be less than 2% at the highest flow rate tested (4 $m\,s^{-1}$).

It is known that cytochrome-*c* unfolds in an acidic medium (*p*H 2.0) and refolds to a molten-globular state with a structure similar to its native state by addition of monovalent ions.[12] The folding kinetics were tested by mixing a 2 mM cytochrome-*c* solution in D_2O at *p*H 2 in one syringe against 400 mM KCl in D_2O in the second syringe. The mixing was

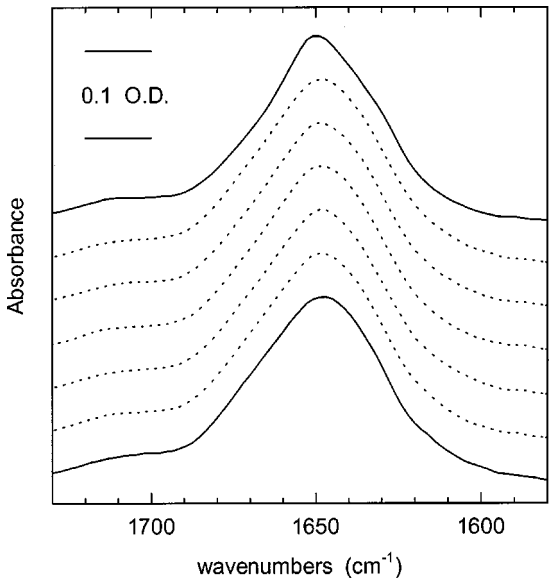

FIG. 2. Time-resolved Amide I band of cytochrome-*c* (dotted lines) and equilibrium data obtained under flow conditions (solid lines). Time-resolved spectra were taken at various points in the cell while mixing 2 mM cytochrome-*c* (*p*H 2) with 400 mM KCl; equilibrium spectra were acquired by premixing the protein with D_2O (bottom curve) or 400 mM KCl (top), and injecting the equilibrium solutions into the observation channel using the syringe pump at the same injection rate as for the time-resolved spectra. 64 scans with 4 cm^{-1} resolution are coadded. The spectra are offset for clarity.

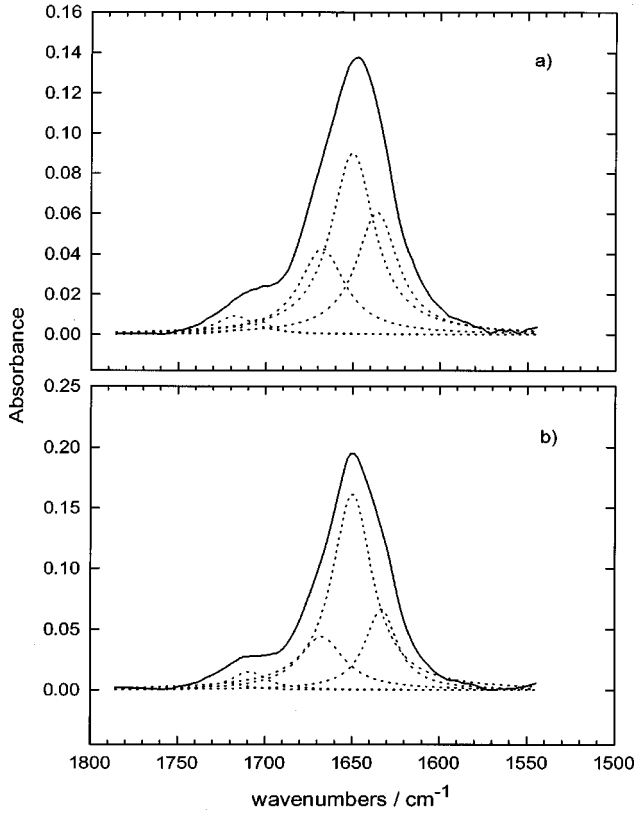

FIG. 3. Spectral deconvolution of the Amide I bands obtained by premixing cytochrome-*c* (*p*H 2) with (a) D_2O, and with (b) 400 mM KCl.

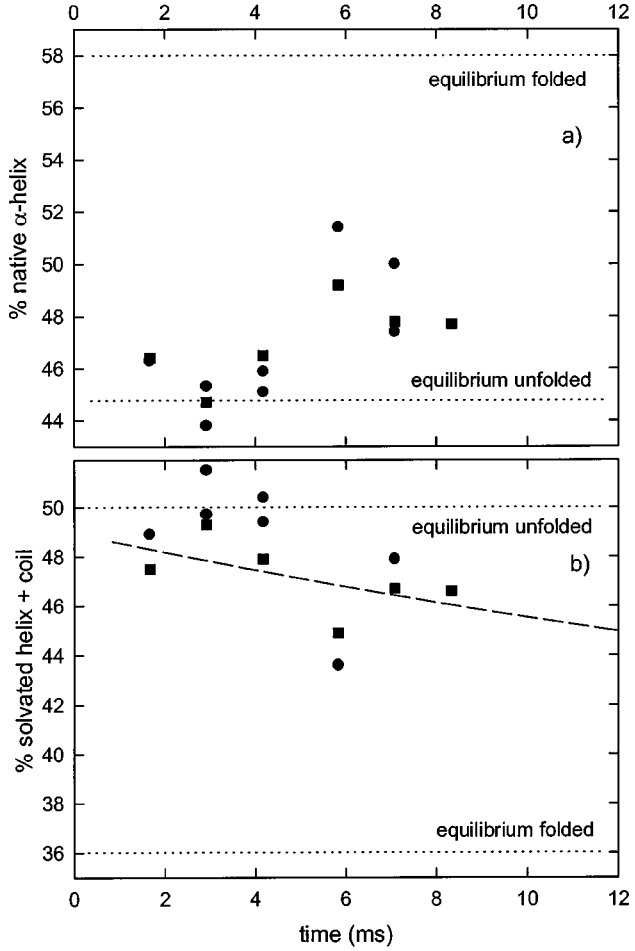

FIG. 4. Time-dependent changes in (a) native α helix, and (b) the sum of coil and solvated α-helix contributions to the total area of Amide I band. A first-order exponential decay function, $y=y_0+\Delta y^* \exp(-t/\tau)$, was fitted through the data points in (b), where y is the percentage of solvated α helix and coil at a time t, y_0 is the percentage of solvated α helix and coil for the equilibrium spectrum of the molten-globular state (36.1%), Δy is the difference in the percentages of solvated α helix and coil between the two extreme (equilibrium) spectra (12.8%), and τ is the time constant for the reaction (32.3 ms).

initiated, and then FTIR data acquisition was started at specific points after the origin of mixing.

Figure 2 shows the time-resolved Amide I band data of cytochrome-*c*, together with two spectra representing the equilibrium endpoints, i.e., the unfolded and molten globule state. As can be seen from the figure, the premixed solution containing cytochrome-*c* at *p*H 2 and 200 mM KCl (final) produces a change in the shape and width of the Amide I band with respect to those in the unfolded equilibrium spectrum (FWHM diminishes from 48 to 43 cm^{-1}). This change is primarily due to loss of coil and solvated α helix at 1666 and 1635 cm^{-1}, respectively (see later). The time-resolved spectra fall between the two limiting (equilibrium) cases.

The signal/noise ratio is better than 100 for 64 scans at 4 cm^{-1} resolution (collection time 45 s), enabling one to accurately perform spectral deconvolution using spectral analysis software (Grams/32, Galactic Industries Corp.). Assuming that the extinction coefficients of the deconvoluted bands are the same,[13] protein secondary structure can be determined as the integrated area contributions of the component band to the total area of the Amide I band. The number of bands in the Amide I envelope was tested by second and fourth derivative analysis of the spectrum. Three peaks were found in the 1700–1600 cm^{-1} region, representing the contributions of coil, native α helix and solvated α helix, with the band maxima at 1666, 1650, and 1635 cm^{-1}, respectively (Fig. 3). These band assignments were proposed recently in the folding of D_2O-solvated myoglobin, a protein with similar secondary structure components as cytochrome-*c*. A significant increase of the native (nonsolvated) α-helix content is observed upon folding. Simultaneously, the contents of solvated α helix and coil diminish. This behavior is expected, because upon folding of the protein helical structure increases as intramolecular hydrogen bonds between C=O and N–H groups of the peptide backbone are established at the expense of hydrogen bonding to the bulk water phase.

Time-resolved secondary structure changes of cytochrome-*c* obtained by spectral deconvolution of the Amide I band are presented in Fig. 4 as changes in content of native α helix versus time, and the change in the sum of coil and solvated α helix of the protein versus time. Obvious trends are observed in the plots. The percentage of α helix in the Amide I band deviates from its content in the unfolded state, steadily increasing with time [Fig. 4(a)]. Under the low *p*H conditions of this experiment, cytochrome-*c* folds rapidly, since the protonated, non-native histidine residues do not ligate to the accessible heme iron atom. Thus, kinetic traps are avoided.[2,6] However, the folding is not completed during the time frame of these measurements. In fact, if one assumes that the content of the native α helix represents a

direct measure of the percentage of cytochrome-*c* that is either folded or unfolded, then after 10 ms only about 30% of the protein is found in the molten-globule state. Similarly, the sum of coil and solvated helix diminishes with time. One can fit the data points in the plot presented in Fig. 4(b) with a first-order exponential decay function to measure the time constant of the folding reaction. The time constant obtained is on the order of 30 ms, which is in agreement with an earlier circular dichroism (CD) study also carried out under acidic conditions.[2]

V. DISCUSSION AND FURTHER DIRECTIONS

We have discussed the fundamentals of the rapid-mixer flow cell design and its implementation in the testing of the cytochrome-*c* folding kinetics. The agreement of the time constant obtained in the present experiments with earlier CD studies shows that the mixer works well in the millisecond time domain. In principle, the shortest time achievable with this technique depends on the spatial resolution of the synchrotron infrared microspectrometer as well as on the dead time of mixing cell. While the former implies a potential for time scales as short as a few microseconds the dead time of the current mixer is on the order of tens of microseconds.[6,9]

In the existing device, the seal between the IR-transparent windows and the mixer is a current limitation that allows examination of the solution about a millimeter from the mixing point. Improvement in the mixer can be achieved by (1) reducing the depth of the mixing well, (2) narrowing the thickness of the seal, and (3) increasing the solution flow rate. This will allow examination of the folding kinetics in the submillisecond regime. Changes in the extent of solvated helix and coil coincident with, and/or subsequent to, collapse of the tertiary structure of cytochrome-*c*, which occurs in the tens of microseconds time scale,[6,9] could then be monitored. On the other hand, the longest achievable time points depend on the ability of the mixer to completely blend the two solutions at low flow rates. At present, the slowest reliable flow rates obtained were on the order of 2 $m\,s^{-1}$. The cell also can be adapted for the longer times by making the grove in ZnSe window thicker, or by cutting a V-shaped channel that opens up towards the exit. Such design should allow studies with time constants of the order of tenths of a second. Finally, the acid-induced folding of other proteins, or the structural changes of other reactions of interest, can be examined with this device.

ACKNOWLEDGMENTS

This study has been supported by the Biomedical Technology Program of the National Center for Research Resources, National Institutes of Health, Grant No. P41RR01633. The National Synchrotron Light Source is supported by the U.S. Department of Energy under Contract No. DE-AC02-98CH10886 to Brookhaven National Laboratory. D.L.R. and S.R.Y. are supported by the National Institutes of Health, Grant Nos. GM4806 and GM54812.

[1] D. N. Brems and E. Stellwagen, J. Biol. Chem. **258**, 3655 (1993).
[2] T. R. Sosnik, L. Mayne, R. Hiller, and S. W. Englander, J. Struct. Biol. **1**, 149 (1994).
[3] H. Roder, G. A. Elove, and S. W. Englender, Nature (London) **335**, 700 (1988).
[4] G. A. Elove, A. F. Chaffotte, H. Roder, and M. E. Goldberg, Biochemistry **31**, 6876 (1992).
[5] R. Gilmashin, S. Williams, R. H. Callender, W. H. Woodruff, and R. B. Dyer, Biochemistry **36**, 15006 (1997).
[6] S. Takahashi, S. R. Yeh, T. K. Das, D. S. Gottfried, and D. L. Rousseau, Nat. Struct. Biol. **4**, 44 (1997).
[7] S. Takahashi, Y.-C. Ching, J. Wang, and D. L. Rousseau, J. Biol. Chem. **270**, 8405 (1995).
[8] R. Gilmanshin, S. Williams, R. H. Challender, W. H. Woodruff, and R. B. Dyer, Proc. Natl. Acad. Sci. U.S.A. **94**, 3709 (1997).
[9] M. C. R. Shastry, S. D. Luck, and H. Roder, Biophys. J. **74**, 2714 (1998).
[10] G. P. Williams, Surf. Sci. **368**, 1 (1996).
[11] W. D. Duncan and G. P. Williams, Appl. Opt. **22**, 2914 (1983).
[12] T. Jordan, J. C. Eads, and T. G. Spiro, Protein Sci. **4**, 716 (1995).
[13] H. Susi, D. M. Byler, and J. M. Purcell, J. Biochem. Biophys. Methods **11**, 235 (1985).